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Cellular Manifestations of Human Papillomavirus Infection in the Oral Mucosa

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Background and Objectives: Human papillomavirus infection has been suggested to play a role in the development of epithelial carcinomas, particularly those of the uterine cervix. Less information is available on the role of the virus in oral lesions. It has been proposed that the viral oncoproteins specifically complex with the products of cellular tumor suppressor gene, namely E6 with p53 and E7 with retinoblastoma gene product. Inactivation or mutation in p53 gene is also known to result in loss of control over the cell cycle and increases in tumor proliferation rates. The present study examines the role of HPV infection in relation to p53 and the extent of the tumor proliferative compartment reflected by cyclin D1 and Ki-67 expression during various phases of tumor progression in the oral epithelium.

Method: Nonisotopic in situ hybridization (NISH) was performed to detect HPV 6/11 and 16/18. Expression of p53, cyclin D1, Ki-67, and the HPV 16/18 E6 protein were detected by immunocytochemistry.

Results: There was significant correlation between the extent of histological abnormality and HPV infection. A correlation ($r = 0.250$, $P = 0.0089$) was evident between the presence of HPV 16 and occurrence of invasive cancer. Expression of the tumor suppressor p53 protein also showed significant positive correlation with histology ($r = 0.475$, $P = 0.00004$). The tumor proliferative fraction also increased with the extent of histological abnormality ($r = 0.387$, $P = 0.0003$ for cyclin D1 and $r = 0.463$, $P = 0.0001$ for Ki 67). Accumulation of p53 and increase in tumor proliferation also correlated to the presence of HPV infection ($r = 0.511$, $P = 0.00003$ for p53; $r = 0.478$, $P = 0.00002$ for cyclin D1 and $r = 0.521$, $P = 0.00004$ for Ki-67).

Conclusions: The present study thus demonstrates the importance of HPV infection in oral tissue. Expression of the high-risk HPV 16/18 E6 protein also appears to be a critical event along with aberrant p53 expression. These results are of significance to the molecular epidemiology of oral cancer and may also be used to supplement and elaborate the diagnosis of oral lesions. *J. Surg. Oncol. 1999;71:10–15.*

KEY WORDS: **p53; cyclin D1; Ki-67; tumor proliferation**

INTRODUCTION

Oral cancer regrettably still has a high mortality rate. The incidence of oral cancer is seen to vary from region to region and some of the highest rates are seen in India, Sri Lanka, Vietnam, Philippines, Hong Kong, and Tai-

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wan, where up to 30% of all cancers are reported to occur in the oropharyngeal region [1]. It is estimated that in India about 56,000 new cases occur each year, resulting in about 100,000 individuals suffering from the disease at any given time [2]. The age-adjusted rates of 24.2/105 for males and 11.2/105 for females in the district of Thiruvananthapuram, Kerala State, India are probably among the highest reported incidence rates in the world [3].

Human papillomaviruses (HPVs) are small DNA viruses that induce proliferation and subsequent disease in squamous epithelium. HPVs exist in two general categories: low-risk types associated with the development of benign lesions, and high-risk types associated with the development of malignant lesions. Evidence for the possible role of HPVs in the development of oral cancer has been previously presented by us [4]. HPV DNA may be found in two different physical states: either as episomes, or integrated into the host genome. Here again, there is a difference between the low-risk and high-risk HPV subtypes. In cervical tissue, the low-risk HPV types 6 and 11 are mostly episomal in low-grade lesions, while types 16 and 18 are usually found integrated into the host genome in high-grade lesions and invasive cancer [5]. These observations appear to be of importance in the process of epithelial tumor progression. Integration implies a disruption for both the host and viral genome which could lead to genetic alterations. In vitro studies have also shown that integration leads to immortalization of cultured cells [6,7]. Quantitative assays demonstrate that there is a progression which occurs even in vitro, characterized by the conversion of large pleomorphic keratinocytes into small, highly proliferative cells [8]. This process is not well defined, but requires the expression of two HPV genes: E6 and E7 [5–7]. Molecular analyses indicate that these viral oncoproteins specifically complex with the products of cellular tumor suppressor genes, namely E6 with p53 and E7 with the retinoblastoma gene product [5–7]. Such interaction could therefore have clinical significance. Cells expressing the E6 protein often have inactive p53 and become resistant to normal regulators of keratinocyte proliferation such as TGFβ [9]. The protein product of p53 gene produced in response to DNA damage acts to restrain cellular proliferation by binding to specific regions of DNA, and regulates the expression of other genes responsible for cell-cycle arrest, DNA repair, and initiation of apoptosis [10]. Inactivation or mutation in p53 gene may therefore result in loss of control over the cell cycle and increases in tumor proliferation rates. It is with this background and rationale that the present study examines the role of HPV infection in relation to p53 and the extent of the tumor proliferative compartment reflected by cyclin D1 and Ki-67 expression during various phases of tumor progression in the oral epithelium—ranging from normal tissue to hyperplasia, dysplasia, and invasive oral cancer.

TABLE I. Distribution of Patients

Histopathology	Subjects
Normal oral mucosa	10
Hyperplasia	22
Dysplasia	16
Invasive squamous cell carcinoma	61
Total	109

MATERIALS AND METHODS

Study Subjects and Tissue Preparation

The study subjects included 61 previously untreated patients with invasive oral cancer and 38 with clinically described leukoplakia lesions. Also analyzed were 10 apparently normal oral mucosa samples. Details of the tissue samples are given in Table I. The study was approved by the Research Advisory Committee of the Regional Cancer Centre. Tissue samples were obtained as incision or punch biopsies during the clinicopathological work up of the patients. Normal oral mucosa was obtained from the patients undergoing dental and orofacial reconstructive surgery. Five micrometer-thick serial sections were cut from paraffin-embedded blocks, deparaffinized, and stained with hematoxylin and eosin. Serial sections were then used for in situ hybridization for HPV evaluation and immunocytochemistry.

DNA–DNA In Situ Hybridization

Nonisotopic in situ hybridization (NISH) was performed to characterize HPV infection using fluorescein-labelled HPV probes specific for types 6/11 and 16/18 (Biogenex, San Ramon, CA), as explained in detail by us earlier [11]. Negative controls included substitution of the probe with saline. Positive controls were smears from the HPV 16-infected cell lines HeLa and CaSKi.

Immunocytochemistry

Sections stained with haematoxylin and eosin were evaluated for each patient, and a representative paraffin block was selected for immunocytochemical studies. Immunocytochemical analysis was performed with an unlabelled primary antibody and the streptavidin–biotin complex method as described by us earlier [12,13]. Sections were incubated overnight with the respective primary antibody p53 (Ab-6), cyclin D1 (Ab-3), Ki-67/MIB-1 (Ab-1), and HPV 16/18 E6 (Oncogene Science, Inc., Cambridge, MA). Negative controls were run omitting the primary antibody. The reaction was visualised using a streptavidin–biotin–immunoperoxidase system (DAKO AS Glostrup, Denmark) with diaminobenzedine as chromogen. All sections were then counterstained with haematoxylin. Negative controls included substitution of the primary antibody with mouse preimmune serum. Positive controls for p53 included a sample of infiltrating duct carcinoma known to have p53 mutation

and express high amounts of p53 protein accumulation. For E6, smears from the HPV 16-infected cell lines HeLa and CaSKi known to express E6 were used as positive controls [14].

Evaluation of p53, Cyclin D1, Ki-67, and E6 Expression

To analyze for the expression of p53, bcl-2, bax, cyclin D1, and Ki-67 protein expression, a total of 1,000 cells were evaluated in all sections. Expression of p53, cyclin D1, and Ki-67 was considered significant when characteristic nuclear immunoreactivity was seen in at least 10% of the tumor cells. In addition to this, an expression index was also created, as done by us earlier [12,13]. This was done by classifying the protein expression into four categories. Thus, grade 1 included those samples with less than 10% positive cells (insignificant); grade 2 included samples showing 11–30% positive cells (mild expression), grade 3 included samples showing 31–50% positive cells (moderate expression), and grade 4, samples showing more than 51% positive cells (intense expression). For E6 expression, only presence or absence of immunoreactivity was considered.

Data Analysis

Statistical analysis by the Kruskal Wallis one-way ANOVA was done to look for relationships between the presence of HPV infection, the presence of the HPV E6 protein, accumulation of p53, expression of cyclin D1 and Ki-67, as well as the extent of histological abnormality. A Spearman correlation was also done to determine any association between type of HPV infection, other cellular parameters shown above E6 expression and stage of tumor progression. For assessing odds ratios (OR), the Fisher's exact test was used. Subjects were divided into two groups based on the extent of histological abnormality. Thus, normal oral tissue and benign lesions (hyperplasia) were grouped as controls, while dysplasia and invasive carcinoma were classified as cases. ORs were calculated as estimates of the relative risk to test for any significant association between presence versus absence of HPV 16. Analysis showing a confidence interval above 95% ($P < 0.05$) were considered significant.

RESULTS

HPV Infection and Expression of the High-Risk HPV E6 Protein

The presence of HPV as assessed by NISH in oral epithelium was clearly evident and is illustrated in Figure 1A. The signal was mostly nuclear, although occasionally it was also seen in both the nucleus and cytoplasm. HPV infection was completely absent in normal oral tissue and hyperplastic lesions. Of the 16 dysplastic samples, two were positive for HPV 6/11 and two positive for HPV 16/18. In invasive squamous cell carcinoma, of the 61 samples, four were positive for HPV 6/11 and 39 positive for HPV 16/18. Details of HPV infection are shown in Figure 1B.

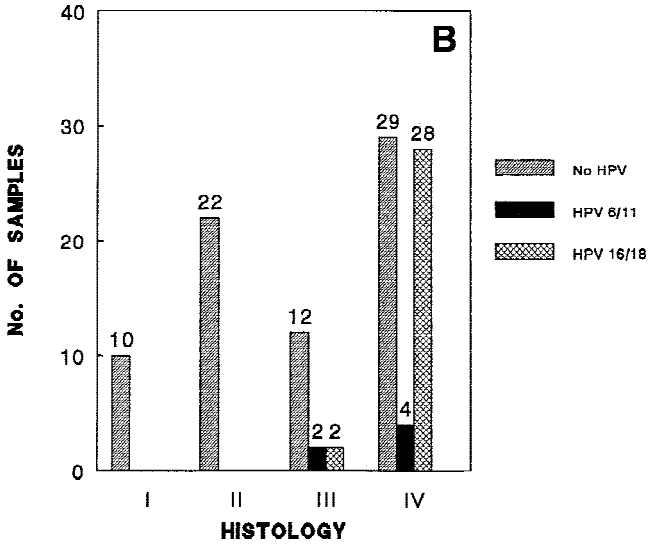

Fig. 1B. See legend on following page.

Immunoreactivity of the high-risk HPV 16/18 E6 protein was mostly cytoplasmic (Fig. 1C), although cases of nuclear expression were seen. Of the two HPV 16/18-infected dysplastic oral tissue, one expressed E6. Of the 39 HPV 16/18-infected invasive cancers, 22 showed presence of the protein. None of the other samples expressed E6. Details on E6 expression are given in Table II.

Immunoreactivity of p53

p53 protein expression was apparent from clear nuclear immunoreactivity in tumor cells (Fig. 1D). The protein was differentially distributed among the various histological grades of lesions. None of 10 normal oral tissue samples were immunoreactive for p53. Of the 22 hyperplastic lesions, one sample expressed moderate levels of p53. Five of the 16 dysplastic cases showed moderate expression, seven showed mild expression, and four showed no immunoreactivity for p53. All 61 invasive carcinomas were immunoreactive for p53, with 38 samples showing moderate expression and 23 samples showing intense expression of p53. Details of p53 protein expression are given in Table III.

The Tumor Proliferative Compartment

The total proliferative status of tissue samples was assessed by the expression of Ki-67 and cyclin D1 (Fig. 1E, F). The results obtained by both methods were similar, though the number of cyclin D1 positive cells was slightly lower than for Ki-67. There was an apparent increase in the proliferative compartment as the lesions

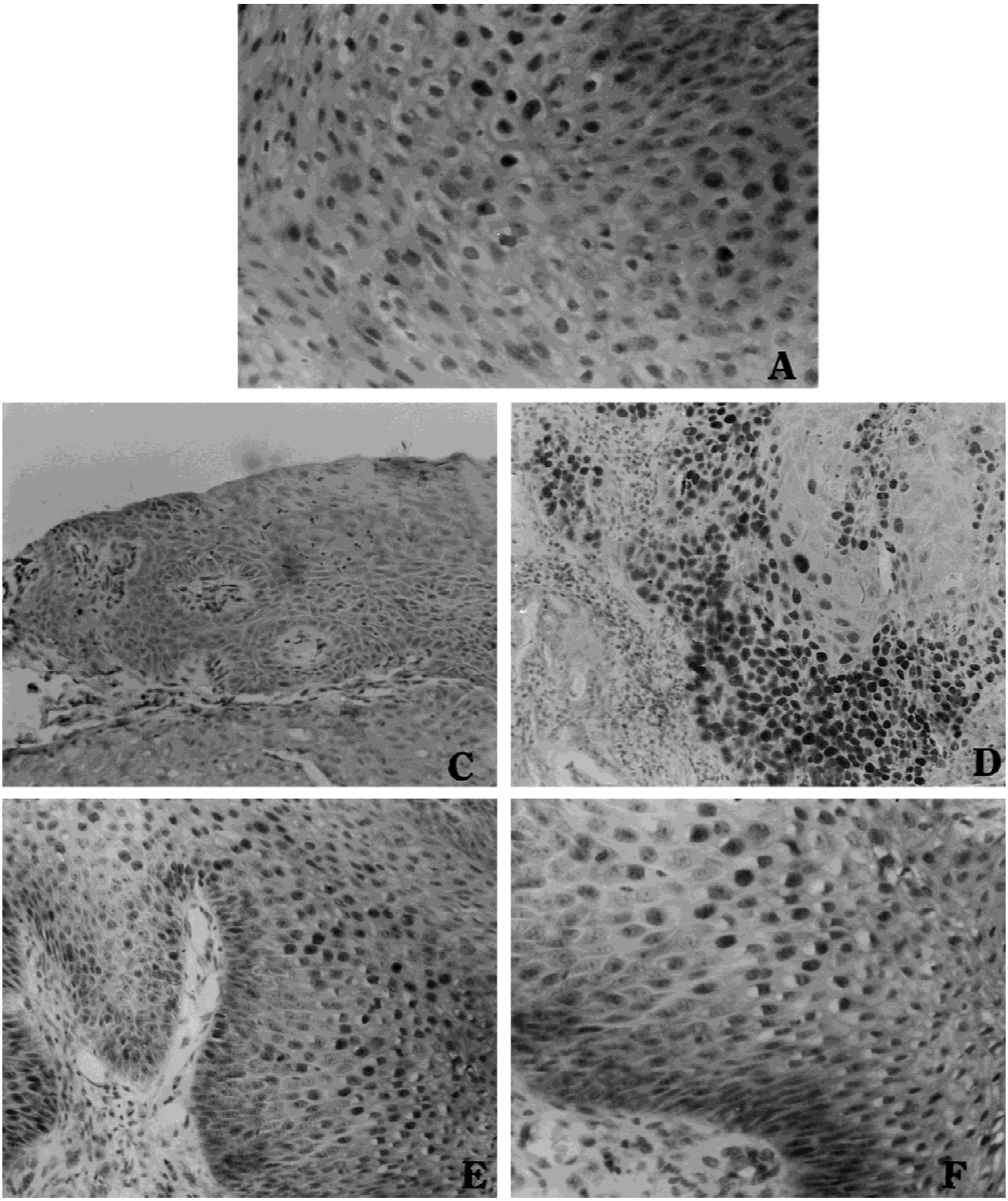

Fig. 1. **(A)** NISH signal generated in squamous cell carcinoma of the oral cavity with a HPV 16/18-specific probe (×425). **(B)** Bar diagram representing the number of cases with various histology showing positivity for HPV infection in oral carcinoma. **(C)** Cytoplasmic localization of HPV E6 protein in oral carcinomas (×225). **(D)** Typical nuclear immunoreactivity of p53 in invasive oral cancer (×365). **(E)** Typical nuclear immunoreactivity of Ki-67 in invasive oral cancer (×365). **(F)** Nuclear immunoreactivity for cyclin D1 protein in invasive oral cancer (×425).

TABLE II. Expression of E6 Protein in HPV 16/18-Infected Oral Tissue

Tissue type	HPV 16/18-infected	E6 positive
Normal (n = 10)	0	0
Hyperplasia (n = 22)	0	0
Dysplasia (n = 16)	2	1
Invasive carcinoma (n = 61)	39	22

progressed through increasing histological abnormality. These results are described in Table III.

Data Analysis

There was significant correlation between the extent of histological abnormality and HPV infection. A correlation ($r = 0.250$, $P = 0.0089$) was evident between the presence of HPV 16 and occurrence of invasive cancer. Expression of the tumor suppressor p53 protein also showed significant positive correlation with histology ($r = 0.475$, $P = 0.00004$). The tumor proliferative fraction also increased with the extent of histological abnormality ($r = 0.387$, $P = 0.0003$ for cyclin D1 and $r = 0.463$, $P = 0.0001$ for Ki-67). Accumulation of p53 and increase in tumor proliferation also correlated to the presence of HPV infection ($r = 0.511$, $P = 0.00003$ for p53; $r = 0.478$, $P = 0.00002$ for cyclin D1; and $r = 0.521$; $P = 0.00004$ for Ki-67).

Fisher's exact test also revealed that when all subjects were grouped either as cases (all dysplasias and invasive cancer) and controls (all normal and hyperplastic oral tissue), the odds ratio of an HPV 16-infected tissue being a case was 0.021 ($P = 0.0001$, 95% Cl:0.001, 0.0356).

DISCUSSION

Molecular analysis of recombinant HPV DNA has provided information on their genomic organization, protein functions, and transcriptional regulation. It has been proposed that integration of the viral genome provides a selective advantage leading to uncontrolled proliferation of the cell due to the deregulated expression of the E6 and E7 genes [5–7]. There is evidence that the expression of the HPV-encoded E6 protein could play a role during malignant progression. The E6 protein encoded by HPV 16 and 18 can interact with the cellular p53 protein and target it for degradation through a ubiquitin-dependent pathway [7,15,16]. Human cells expressing these E6 proteins show a significant reduction in the stability of newly synthesized endogenous p53 [6,7,15,16]. In contrast, the E6 protein encoded by low-risk HPV 6 and 11 show a reduced binding to p53 and are unable to target degradation of this protein in vitro [15]. The present study shows an obvious association between accumulation of p53 with both HPV 16/18 infection and E6 protein expression. E6 can interfere with the normal functioning of p53 by its ability to abrogate both transcriptional activation and transcriptional repression function of the gene [7,15,16]. In this respect, E6 shows similarity to certain mutant forms of p53 which also fail to function as transcriptional regulators themselves and may also inactivate the wild-type p53 after oligomerisation [7]. There is data to suggest that the HPV E6 protein may also function in inhibiting apoptosis in certain cells [7,17]. p53 has been shown to be required for apoptosis induced by adenovirus EIA, ionising radiation, and etoposide [10]. The loss of function of the p53 check point regulator due to its interaction with high-risk HPV-E6 may thus impair the apoptotic response to virally infected cells. We have previously reported the biological consequences of E6-p53 interaction in cervical cancer cells including modulation of apoptotic regulatory molecules [18]. Since p53 is a negative regulator of proliferation, this could also explain the higher rates of tumor proliferation as reflected by increased cyclin D1 and Ki-67 expression. Recent data [19] also shows that the E6 protein can interact with cellular MCM proteins, which are believed to have a key role in regulation of DNA replication. The correlation between the avidity of interaction of the E6 protein with p53 and the apparent oncogenic activity of the virus suggests that this activity of E6 may contribute to the malignant potential of the high-risk HPV. Thus, HPV infection defined by the presence of HPV DNA may not be sufficient to evaluate tumor progression. The argument is that the presence of DNA alone may not be enough to cause active infection because it could be episomal or otherwise nonproductive. E6 protein expression would therefore be a necessary requirement for HPV activity.

The antibody used in the present study for the detection of p53 can detect both mutant and wild-type p53 protein. Wild p53 protein has a short half-life (6–20 min) but mutant forms have a half-life up to 6 hr. Thus, detection of p53 by immunocytochemistry is often considered to reflect the mutant form [10,20], although this may not be true in all cases, since immunocytochemical analysis of p53 protein may vary according to the antibody used—an issue we have stressed earlier [21]. The function of normal p53 protein can be attenuated by the E6 of high-risk human papillomaviruses (or possibly by other endogenous proteins such as mdm-2), resulting in a functionally inactive form. The strong positive correlation observed between p53 detected by immunocytochemistry and extent of histological abnormality could also be on account of the higher synthesis of the p53 protein as a result of excessive DNA damage, accumulation of the E6–p53 complex, or due to some factor prolonging the half-life of the p53 protein, allowing its detection by immunocytochemistry. However, mutations in p53 are frequent in oral cancer and result in the production of a functionally defective p53 protein [22]. We have recently also demonstrated specific accumulation of

TABLE III. Intensity of p53, KI 67, and Cyclin D1 Expression in Oral Tissue*

	p53 intensity (classes)				Ki-67 intensity (classes)				Cyclin D1 intensity (classes)			
Tissue type	1	2	3	4	1	2	3	4	1	2	3	4
Normal (n = 10)	10	—	—	—	10	—	—	—	10	—	—	—
Hyperplasia (n = 22)	21	—	—	—	20	2	—	—	—	19	3	—
Dysplasia (n = 16)	4	7	5	—	—	2	13	1	—	3	12	1
Invasive carcinoma (n = 61)	—	—	38	23	—	—	12	49	—	—	32	29

*Values are expressed as percents: Class 1, 0–10% positive cells; class 2, 11–30% positive cells; class 3, 31–50% positive cells; class 4, >51% positive cells.

mutant p53 in oral lesions [23]. Mutant p53 protein can attain stable conformation by forming dimers or tetramers and accumulating in the cell [20].

The factors involved in the progression of precancerous processes in the oral cavity to invasive cancer continues to be an area of intense investigation. The issue is critical since it addresses the biological characteristics that typify neoplasia, and the possibility that such information will simplify the management of patients with premalignant lesions. Cellular and molecular characteristics need to be identified and evaluated in context of clinical significance. It is known that lesions at any point in the premalignant spectrum have been associated with subsequent invasion—reflecting the limitations of histological grading for predicting the risk of malignant progression. It was with these limitations in mind that the American Joint Committee on Cancer had called for a biological staging system to be integrated with existing clinical and pathological grading systems [24]. The present study shows the importance of HPV infection in oral tissue. These results are of significance to the molecular epidemiology of oral cancer and may also be used to supplement and elaborate the diagnosis of oral lesions. Such a unified approach may also provide the basis for the identification of high-risk premalignant lesions, may provide potential targets for intervention, and, in addition, may provide surrogate endpoint biomarkers for chemopreventive approaches.

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